CHAPTER-4

EXPERIMENTAL INVESTIGATIONS

4.1 SELECTION OF ANTIRETROVIRAL DRUGS (RITONAVIR, ATAZANAVIR AND INDINAVIR) AND ANTI-DIABETIC DRUG (REPAGLINIDE) DOSES

In clinical practice antiretroviral drugs especially protease inhibitors causes diabetes mellitus on long term usage, to treat this oral anti-diabetic drug like repaglinide is used HIV patients. Human oral therapeutic doses of the respective drugs were calculated to rat dose considering body surface area. However the dose of repaglinide for rat experiment was selected based on the body weight (0.5mg/kg) which effects the dose relationship of repaglinide on blood glucose in rats [66].

Table 4.1: Selection of antiretroviral drugs doses for the studies

<table>
<thead>
<tr>
<th>S.No</th>
<th>Drug</th>
<th>Human therapeutic doses</th>
<th>Rats dose (mg/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ritonavir [67]</td>
<td>100-200mg boosting dose</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>Atazanavir [68]</td>
<td>400mg once daily</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>Indinavir [69]</td>
<td>800mg oral every 8hrs</td>
<td>72</td>
</tr>
</tbody>
</table>

4.2 PREPARATION OF FORMULATION

The required amounts of the above drugs in combinations (Repaglinide plus Ritonavir, Repaglinide plus Atazanavir and Repaglinide plus Indinavir were weighed and placed in separate mortars triturated with pestle, to this required amount of tween 80 (1%) was added as a wetting agent and in order to ensure that the whole compound becomes wet. By using gravimetric dilution method, 0.5% of the methyl cellulose was added and triturated until the uniformity was achieved [70].
35

4.3 ADMINISTRATION OF FORMULATIONS TO WISTAR RATS

Male albino Wistar rats weighing 200-250 gm were obtained from the Mahaveera enterprises (Hyderabad, India). Under standard conditions, following a 12 hour light/dark cycle the animals were kept in the laboratory of Creative Educational Society’s college of pharmacy, Kurnool, India. Until before dosing (over night) and 4 hours after dosing rats were on fasting. Water was allowed *ad-libitum* during the fasting period. Prior approval of the study protocol was obtained from the institutional animal ethics committee (1305/ac/09/CPCSEA). For the purpose of control and supervision of experiments on animals (CPCSEA), the experiments were conducted according to the guidelines provided by the committee. The total 120 rats were used for pharmacokinetic and pharmacodynamic study; these groups were indicated as pharmacokinetic interaction study group as PK group and pharmacodynamic interaction study group as PD group and these rats were used for three combinations (I. Repaglinide plus ritonavir, II.Repaglinide plus atazanavir and
III. Repaglinide plus indinavir. Schematic representation of pharmacokinetic and pharmacodynamic study groups was presented in figure 4.1 and 4.2

Figure 4.2: Schematic representation of administration of drugs to the rats
Pharmacokinetic interaction study in Wistar rats

Combination-I (Repaglinide plus Ritonavir)
- PK-I- Repaglinide alone (Control) (n=6)
- PK-II-Repaglinide in the Pretreated Ritonavir in normal rats (n=6)
- PK-III- Repaglinide in the Pretreated Ritonavir in diabetic rats (n=6)
- PK-IV- Repaglinide in the Pretreated Ritonavir in Hepatic impaired rats (n=6)

Combination-II (Repaglinide plus Atazanavir)
- PK-V-Repaglinide in the Pretreated Atazanavir in normal rats (n=6)
- PK-VI- Repaglinide in the Pretreated Atazanavir in diabetic rats (n=6)
- PK-VII- Repaglinide in the Pretreated Atazanavir in Hepatic impaired rats (n=6)

Combination-III (Repaglinide plus Indinavir)
- PK-VIII-Repaglinide in the Pretreated Indinavir in normal rats (n=6)
- PK-IX- Repaglinide in the Pretreated Indinavir in diabetic rats (n=6)
- PK-X- Repaglinide in the Pretreated Indinavir in Hepatic impaired rats (n=6)

Figure 4.3: Schematic representation of groups divided for the pharmacokinetic studies.
Figure 4.4: Schematic representation of groups divided for the pharmacodynamic studies.
4.4 INDUCTION OF DIABETES MELLITUS IN WISTAR RATS BY USING ALLOXAN

4.4.1 Alloxan Properties

Alloxan is a chemical compound used to induce the diabetes mellitus in research causing necrosis of the β-cells of pancreatic islets selectively.

4.4.2 Mechanism of action

Alloxan reacts with two -SH groups in the sugar binding site of glucokinase and results in inactivation of the enzyme. Dialuric acid is formed and re-oxidized back to alloxan which generates reactive oxygen species (ROS) and superoxide radicals [71].

4.4.3 Procedure

Diabetes mellitus was induced in rats using alloxan monohydrate in ice cold normal saline. Two doses, 100mg and 50 mg/kg body wt administered intraperitoneally for two consecutive days. By retro-orbital plexus, blood samples were withdrawn from rats of all surviving animals and glucose levels in serum were estimated after 72 hrs. Rats with blood glucose levels of 200 mg/dL and above were marked as diabetic and selected for the study [72].

4.5 INDUCTION OF HEPATIC IMPAIRMENT IN WISTAR RATS BY USING CARBON TETRACHLORIDE (CCl₄)

A number of CCl₄ models are available for the induction of liver failure in animals. Liver failure is induction is two types

1. Acute liver failure
2. Chronic liver failure

1. Acute liver failure:

Ischemia, hydropic degeneration and central necrosis are indicators for acute liver damage induced by oral or subcutaneous or intraperitoneally administration of CCl₄ (1-2 ml/kg). Maximum increase of biochemical parameters were noticed after 24 hr of CCl₄ administration which was mixed with equal proportions of liquid paraffin or olive oil (50% v/v).
2. Chronic liver failure

a. Chronic reversible liver failure:
Chronic, reversible liver damage was induced by the administration of CCl₄ (1ml/kg, subcutaneously) two times in a week and repeating the same for 8 weeks.

b. Chronic, irreversible liver failure:
The same procedure was followed as that of reversible liver failure but only difference was administration for 12 weeks instead of 8 weeks.

4.5.1 Mechanism of action
Carbon tetrachloride is metabolized by CYP2E1, CYP2B and possibly CYP3A to form trichloromethyl radical which attacks the polyunsaturated fatty acids of the membrane of the ER, initiating a chain reaction. Cells present in the Centrilobular region where microsomal enzyme activity is greater, are the first to be damaged. The initial damage produced is highly localised in the endoplasmic reticulum which results in the loss of cytochrome P450 turn leads to functional failure lowering protein synthesis and accumulation of triglycerides causing fatty liver[73].

4.5.2 Procedure
Hepatic impairment was induced in rats by administration of CCl₄ diluted in olive oil (2ml/kg) intraperitoneally in 1:1 dilution for one day. After 24hr, blood samples were collected from rats by retro-orbital plexus of all surviving animals and the serum was analysed for total bilirubin (>2mg/dL), alanine transaminase (>150 U/L), aspartate transaminase (>200 U/L) and albumin (<3 g/dL) were selected for the study. [74, 75].

4.6 COLLECTION OF BLOOD SAMPLES
Blood collected by retro-orbital puncture at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h from PK groups and processed to obtain plasma by centrifugation at 8000 rpm for 10 min. Blood samples were collected from PD groups at same time points by retro-orbital puncture and serum was separated by using Remi research centrifuge at 4000 rpm for 15 min. The temperature at which samples were stored in vials was at -80°C until LC/MSMS analysis and the serum samples were analysed for blood glucose estimation at respective time points [76].
Table 4.2: Materials used for collection of blood samples and preparation of formulation

<table>
<thead>
<tr>
<th>S.No</th>
<th>Chemicals</th>
<th>Manufacture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hematocrit heparinised rat bleeding capillaries</td>
<td>Top-Tech solutions, Hyderabad</td>
</tr>
<tr>
<td>2</td>
<td>Eppendorf centrifuge vortex mixer</td>
<td>Remi laboratories instruments, Mumbai</td>
</tr>
<tr>
<td>3</td>
<td>Micro centrifuge tubes 1.5ml capacity</td>
<td>Eppendorf</td>
</tr>
<tr>
<td>4</td>
<td>Absorbent cotton</td>
<td>Jajoo surgical Pvt limited, Madhya Pradesh</td>
</tr>
<tr>
<td>5.</td>
<td>Tween 80</td>
<td>Merck chemicals, Mumbai</td>
</tr>
<tr>
<td>6.</td>
<td>Methyl cellulose</td>
<td>TCI Chemicals (India) Pvt. Ltd., Chennai</td>
</tr>
</tbody>
</table>

4.7 ESTIMATION OF BIOCHEMICAL PARAMETERS

4.7.1 Estimation of serum glucose

4.7.2 Estimation of Total bilirubin (TB)

4.7.3 Estimation of SGOT (AST)

4.7.4 Estimation of SGPT (ALT)

4.7.5 Estimation albumin
4.7.1 Estimation of serum glucose [77]

Serum glucose was estimated by using glucose kit.

Method: Colorimetric method

Principle:

\[
\text{Glucose} + \text{Oxygen} + \text{Water} \xrightarrow{\text{glucose oxidase}} \text{Gluconic acid} + \text{Hydrogen peroxide}
\]

\[
\text{Hydrogen peroxide} + \text{Phenol} + 4\text{-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinonimine} + \text{H}_2\text{O}_2
\]

Table 4.3: Reagent composition:

<table>
<thead>
<tr>
<th>Reagent no</th>
<th>Reagent</th>
<th>Composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose (S.L)R1 reagent</td>
<td>Tris buffer (pH7.4)</td>
<td>92 mmol/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phenol</td>
<td>0.3 mmol/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucose oxidase</td>
<td>15000 cU/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-Amino phenzone</td>
<td>2.6mmol/L</td>
</tr>
<tr>
<td>2</td>
<td>Glucose standard</td>
<td>Glucose standard concentration</td>
<td>100mg/dL</td>
</tr>
</tbody>
</table>

Table 4.4: Procedure for the estimation blood glucose

<table>
<thead>
<tr>
<th>Working reagent</th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1mL</td>
<td>1mL</td>
<td>1mL</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td>0.01mL</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td></td>
<td>-</td>
<td>0.01mL</td>
</tr>
</tbody>
</table>

Mix and incubate at 37°C for about 10 minutes. Observe the absorbance at 500 nm for sample and standard by using the blank reagent.
4.7.2 Estimation of Total bilirubin [78]

Total bilirubin was estimated by In-vitro quantitative determination of bilirubin in serum

**Method:** In-vitro quantitative determination – Modified DMSO method

**Principle:** Diazotized sulfanilic acid was formed when sulfanilic acid reacts with sodium nitrite. Azobilirubin was the product obtained due to the reaction between total bilirubin and diazotized sulfanilic acid.

**Table 4.5: Reagent composition:**

<table>
<thead>
<tr>
<th>Reagent no</th>
<th>Reagent</th>
<th>Composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total bilirubin reagent</td>
<td>Sulfanilic acid HCl DMSO</td>
<td>28.9 mmol/L 165 mmol/L 7 mmol/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Preservatives and stabilizers</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Total bilirubin activator</td>
<td>Total bilirubin activator</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Bilirubin artificial standard</td>
<td>Standard bilirubin concentration</td>
<td>10 mg/dL</td>
</tr>
</tbody>
</table>

**Table 4.6: Procedure for the estimation total bilirubin**

<table>
<thead>
<tr>
<th></th>
<th>Reagent blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bilirubin reagent</td>
<td>1mL</td>
<td>1mL</td>
</tr>
<tr>
<td>Activator total</td>
<td>-</td>
<td>0.02mL</td>
</tr>
<tr>
<td>Serum</td>
<td>0.05mL</td>
<td>0.05mL</td>
</tr>
</tbody>
</table>

Mix thoroughly and incubate for exactly 5 minutes and measure the absorbance of serum against blank at 546 nm.

4.7.3 Estimation of Aspartate Aminotransferase (AST or SGOT)) [79]

SGOT was estimated by In-vitro quantitative determination
**Method:** In-vitro quantitative determination.

**Principle:** Kinetic determination of aspartate amino transferase is based upon the following reaction

\[
\text{L-Aspartate} + \text{Alpha-ketoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L – Glutamate}
\]

\[
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L – Malate} + \text{NAD}^+
\]

---

**Table 4.7: Reagent composition**

<table>
<thead>
<tr>
<th>Reagent no</th>
<th>Reagent</th>
<th>Composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SGOT (S.L) R₁</td>
<td>Tris buffer (pH7.8) L – Aspartate</td>
<td>88mmol/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LDH</td>
<td>260mmol/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MDH</td>
<td>&gt;1500U/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;900U/L</td>
</tr>
<tr>
<td>2</td>
<td>SGOT (S.L) R₂</td>
<td>Alpha ketoglutarate NADH</td>
<td>12mmol/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.24mmol/L</td>
</tr>
</tbody>
</table>

**Table 4.8: Procedure for the estimation of Aspartate Aminotransferase**

<table>
<thead>
<tr>
<th>Working reagent</th>
<th>1mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>0.1mL</td>
</tr>
</tbody>
</table>

Mix thoroughly and incubate at 37°C for 1 minute and then measure change in absorbance per minute (delta OD/minute) during 3 minutes.

---

**4.7.4 Estimation of Alanine Aminotransferase (ALT or SGPT)[80]**

SGPT was determined by In-vitro quantitative determination

**Method:** In-vitro quantitative determination.

**Principle:** kinetic determination of alanine amino transferase according to following reaction

\[
\text{L-Alanine} + \alpha \text{ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L – Glutamate}
\]

\[
\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L – Lactate} + \text{NAD}^+
\]
Table 4.9: Reagent composition

<table>
<thead>
<tr>
<th>Reagent no</th>
<th>Reagent</th>
<th>Composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SGPT (S.L) R$_1$</td>
<td>Tris buffer (pH 7.5) L – Alanine LDH</td>
<td>110 mmol/L 600 mmol/L &gt;1500 u/L</td>
</tr>
<tr>
<td>2</td>
<td>SGPT (S.L) R$_2$</td>
<td>Alpha ketoglutarate NADH</td>
<td>16 mmol/L 0.24 mmol/L</td>
</tr>
</tbody>
</table>

Table 4.10: Procedure for the estimation of Alanine Aminotransferase

<table>
<thead>
<tr>
<th>Working reagent</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1mL</td>
<td>0.1mL</td>
</tr>
</tbody>
</table>

Mix thoroughly and incubate at 37°C for one minute and then measure change in absorbance per minute (delta OD/minute) during 3 minutes.

4.7.5 Estimation of Albumin[81]

Albumin was estimated by using In-vitro quantitative determination.

**Method:** Direct biuret method

**Principle:**

The albumin binds to bromocresol green (BCG), an anionic dye, under acidic pH produce a color change in indicator from yellow –green to green –blue.

\[
\text{Albumin} + \text{Bromocresol green (pH 4.2)} \rightarrow \text{Bromocresol green-albumin complex}
\]

Table 4.11: Reagent composition

<table>
<thead>
<tr>
<th>Albumin reagent</th>
<th>2x50ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate Buffer pH (4.2)</td>
<td>75 mmol/L</td>
</tr>
<tr>
<td>Bromocresol green</td>
<td>0.14 g/L</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Albumin standard</th>
<th>1X3ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin standard concentration</td>
<td>3gm/dL</td>
</tr>
</tbody>
</table>
Table 4.12: Procedure for the estimation of Albumin

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1mL</td>
<td>1mL</td>
<td>1mL</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>0.010mL</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>0.010mL</td>
</tr>
</tbody>
</table>

Mix and incubate for 1 minute. Measure the absorbance of standard and sample against reagent blank.

4.8 LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY ANALYSIS

4.8.1 SAMPLE PREPARATION

4.8.1.1 REPAGLINIDE STOCK SOLUTION

2mg of repaglinide was weighed and transferred to 2ml eppendorf tube. To this 500µl of dimethyl sulfoxide (DMSO) was added and sonicated to aid dissolution and make up the volume with DMSO up to 1ml. This eppendorf tube was sealed with parafilm and stored in refrigerator (2-8 °C).

4.8.1.2 ROSUVASTATIN (Internal Standard Stock Solution)

2mg of rosuvastatin was weighed and transferred to 2ml eppendorf tube. To this 500µl of dimethyl sulfoxide (DMSO) was added and sonicated to aid dissolution and followed by make up the volume with DMSO up to 1 ml. This eppendorf tube was sealed with parafilm and stored in refrigerator (2-8 °C).

4.8.2 TUNING OF MOLECULES

- From the stock solutions 500 ng/ml of repaglinide and 400 ng/ml of rosuvastatin (IS) in acetonitrile was prepared. These molecules were tuned to establish the stable and intense ion beam by optimizing LC parameters and to confirmed the parent ion mass.

- Quantification was achieved with MS-MS detection in positive ion mode for both the analytes and the internal standard using mass spectrometer equipped with a Turboionspray interface at 500 °C.
• Detection of the ions was carried out in the Positive-ion multiple-reaction monitoring mode (MRM) and the selected [M+H]+ precursor ions were m/z 453.2 for repaglinide and m/z 482.3 for rosuvastatin.

• The product ions monitored were at m/z 230.3 and 258.15 for repaglinide, and rosuvastatin (internal standard) respectively. The analysis data obtained were processed by Analyst software™ (version 1.5.1).

4.8.3 PREPARATION OF CALIBRATION CURVE STANDARDS AND QUALITY CONTROL SAMPLES

Calibration curve standard solutions of repaglinide in blank plasma were prepared by spiking with an appropriate volume of the working solutions, giving final concentrations of 9, 19, 39, 78, 156, 312, 625, 1250, 2500, 5000, 10000 and 20000 ng/ml of repaglinide and 400 ng of rosuvastatin.

Quality control samples were prepared at three different concentration levels 29 ng/ml (LQC) 6000 ng/ml (MQC) 15000 ng/ml (HQC) for repaglinide in blank plasma. All the Prepared plasma samples were stored at -70 °C [82].
4.8.4 SAMPLE PROCESSING (Extraction Procedure)

Retrieved the plasma samples from the deep freezer and thawed in water bath maintained at room temperature, vortexed to get uniform mixing of plasma.

Place 30 µl of plasma in eppendorf tubes

To this add 150 µl of Acetonitrile containing internal standard (400ng/ml) was added

Vortexed for one minute and followed by centrifugation at 10000 rpm at 10 °C for 10 minutes

After centrifugation 20 µl of supernatant was transferred to HPLC vial and 5 µl was subjected to chromatographic analysis.

**Figure 4.5 Schematic presentation of extraction of plasma samples**
Table 4.13 Optimized Chromatographic conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile Phase</td>
<td>Acetonitrile : 0.1% formic acid</td>
</tr>
<tr>
<td>Buffer</td>
<td>0.1 % formic acid</td>
</tr>
<tr>
<td>Mode (Isocratic/Gradient)</td>
<td>Gradient</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.3 ml/ min</td>
</tr>
<tr>
<td>Run time</td>
<td>6 minutes</td>
</tr>
<tr>
<td>Column oven temperature</td>
<td>40±2 °C</td>
</tr>
<tr>
<td>Volume of injection</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

4.8.5 Method validation

The validation of the above method was carried out as per US FDA guidelines [83]. The parameters determined were

1. Selectivity
2. Sensitivity
3. Matrix effect
4. Linearity
5. Precision and Accuracy
6. Recovery
7. Stability

4.8.5.1 Selectivity

Selectivity was approached by comparing the chromatograms of six different batches of blank plasma obtained from six different sources including one hemolyzed plasma.
4.8.5.2 Sensitivity

Sensitivity was determined by analyzing six replicates of plasma samples spiked with the lowest level of the calibration curve concentrations.

4.8.5.3 Matrix effect

This was checked with six different lots of disodium-EDTA plasma. Three replicate samples each of LQC and HQC were prepared from different lots of plasma.

4.8.5.4 Linearity

For checking the linearity standard calibration curves containing at least 12 points (non-zero standards) were plotted 9.8-20000 ng/mL for repaglinide. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences.

4.8.5.5 Precision and Accuracy

Inter-day precision and accuracy were determined by analyzing six replicates at three different QC levels on two different days.

Intra-day precision and accuracy were determined by analyzing six replicates at three different QC levels of five different runs.

4.8.5.6 Recovery

Recoveries of the drugs were determined by comparing the peak area of extracted analyte standard with the peak area of non-extracted standard.

Recoveries of Repaglinide was determined at a concentration of 29 (LQC), 6000 (MQC) and 15000 (HQC) ng/mL, respectively, where as for internal standard was determined at concentration of 400 ng/ml

4.8.5.7 Stability tests

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2-8°C) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution.
1. Bench top stability (14 h),
2. Processed samples stability (Auto sampler stability for 60 h)
3. Wet extract stability for 55 h and reinjection stability for 36 h
4. Freeze-thaw stability (4 cycles),
5. Long-term stability (52 days) was performed at LQC and HQC levels using six replicates at each level.
   - Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15% SD) and precision (±15% RSD) [84, 85, 86].

4.9 PHARMACOKINETIC, PHARMACODYNAMIC AND STATISTICAL ANALYSIS

4.9.1 Pharmacokinetic Analysis
Pharmacokinetic parameters were calculated by subjecting the repaglinide plasma concentration-time data to non-compartmental analysis using WinNonlin (Phoenix - v6.3.0; Pharsight, Mountain view, CA) software.

4.9.2 Pharmacodynamic analysis
Pharmacodynamic analysis was carried out by calculating the percentage blood glucose reduction at each time point [87].

Percentage reduction in blood glucose level

\[ \text{Percentage reduction} = \frac{\text{Initial blood glucose level} - \text{final blood glucose level}}{\text{Initial blood glucose level}} \times 100 \]

All the data was presented in mean±standard deviation (SD). The significance was determined by applying one way ANOVA analysis.